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Current Opinion

Insights and controversies into the role of the key apicomplexan invasion ligand, Apical Membrane Antigen 1

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ABSTRACT

Apicomplexan parasites are obligate intracellular pathogens that cause a host of human and animal diseases. These parasites have developed a universal mechanism of invasion involving formation of a 'moving junction' that provides a stable anchoring point through which the parasite invades host cells. The composition of the moving junction, particularly the presence of the protein Apical Membrane Antigen 1 (AMA1), has recently been the subject of some controversy. In this commentary we review findings that led to the current model of the moving junction complex and dissect the major conflicts to determine whether a substantial reassessment of the role of AMA1 is justified.

1. Introduction

Apicomplexan parasites represent an important and diverse group of human and animal pathogens, which includes the causative agents of malaria (*Plasmodium* spp.) and toxoplasmosis (*Toxoplasma gondii*). These obligate intracellular parasites have complex life cycles that encompass a succession of developmental stages, often across multiple host species, and they have evolved highly specialised machinery to actively invade host cells with remarkable co-ordination and speed.

Invasive 'zoites' utilise numerous specific ligands to recognise and invade susceptible host cells. *Toxoplasma gondii* tachyzoites are capable of invading a wide variety of cell types that express vastly different surface receptors. In contrast, *Plasmodium* sporozoites and merozoites are highly selective for hepatocytes and red blood cells, respectively, and merozoites from different *Plasmodium* spp. have varying capacities to invade mature erythrocytes (normocytes). Yet despite apicomplexan parasites having specificity for different host cells, the kinetics and molecular aspects of invasion appear to be conserved over a large evolutionary distance within the phylum (Dvorak et al., 1975; Gilson and Crabb, 2009; Sharma and Chitnis, 2013). This points to a pivotal core mechanism that allows these parasites to maintain comparable invasion efficiency.

2. A universal host cell binding mechanism

Five years ago, Besteiro et al. (2009) proposed a remarkable mechanism whereby parasites supply both ligand and receptor to form an intimate membrane junction between the host and parasite during invasion. This junction, first observed over 30 years ago, is described as an electron-dense interface at the point of contact between the parasite and host cell that encircles and migrates down the length of the parasite during internalisation (Aikawa et al., 1978; Riglar et al., 2011). The discovery by Besteiro et al. that a complex of proteins is secreted into the host side of this so-called 'moving junction' (MJ) was a key insight into this unique mechanism of host cell penetration. Formation of an entirely parasite-derived host-anchoring complex would allow parasites to rely less upon the host and thus maintain invasion efficiency across different host cell types. Species-specific adhesins could act upstream of this mechanism to identify a susceptible host cell (reviewed in Harvey et al., 2012), before a conserved multiprotein adhesin complex is deployed to maintain the high level of coordination that is observed across all parasites within the phylum.

Apical Membrane Antigen 1 (AMA1), a micronemal integral membrane protein, is the putative ligand in the MJ, and a complex of rhoptry neck-derived (RON) proteins, RON2, RON4 and RON5 (and RON8 in *T. gondii*), appears to translocate into the host cell to act as a receptor for AMA1.

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2.1. Evidence to support the role of AMA1 and RON proteins in the MJ complex

Binding of AMA1 to the RON protein complex was first observed by Alexander et al. (2005) and several studies have since validated this interaction (Alexander et al., 2006; Besteiro et al., 2009; Cao et al., 2009; Collins et al., 2009; Lebrun et al., 2005; Narum et al., 2008; Richard et al., 2010). AMA1 has a large N-terminal ectodomain that is structurally conserved across genera, folding into three interacting domains with several protruding loops (Bai et al., 2005; Crawford et al., 2010; Hodder et al., 1996; Pizarro et al., 2005). RON2 adopts a membrane-spanning conformation such that a C-terminal loop region is exposed on the surface of the host cell (Lamarque et al., 2011; Srinivasan et al., 2011). RON4 and RON5 (and RON8) have no transmembrane regions and appear to localise entirely within the host cell cytosol to interact with the host cytoskeleton (Riglar et al., 2011; Srinivasan et al., 2011; Takemae et al., 2013). This complex could provide a physical link between the cortical cytoskeletons of both cells to serve as a stable anchoring structure upon which the zoite can apply traction. Tonkin et al. (2011) and Hossain et al. (2012) mapped in detail the binding interface between AMA1 and RON2 in *T. gondii* and *Plasmodium falciparum*, respectively. A hydrophobic trough within domain I of AMA1 forms a binding pocket that accepts the critical loop region in RON2 with significant shape and charge complementarity. In silico modelling illustrates that RON2 displaces a loop in domain II of AMA1 to expose the binding surface, then the exposed RON2 loop can penetrate deep within the hydrophobic groove in AMA1 (Tonkin et al., 2011). This high affinity association would likely withstand mechanical forces and, as such, further supports the role for AMA1-RON complex binding to maintain close contact with the host cell during active invasion.

While the molecular composition of the MJ has been challenging to resolve, largely due to its transient existence over a fleeting internalisation period, there is now considerable evidence to support a role for AMA1 and the RON protein complex in the MJ. Anti-AMA1 antibodies and competitive binding peptides that block the interaction between AMA1 and RON2 inhibit invasion at the stage of MJ formation, when parasites can no longer form intimate contact with the host cell (Treeck et al., 2009; Richard et al., 2010; Srinivasan et al., 2011). Most notably, immunostaining of invading *T. gondii* tachyzoites and *P. falciparum* merozoites demonstrates that the RON complex is located on the host side of the MJ and that the majority of surface-bound AMA1 co-localises with the RON complex in the MJ plane (Alexander et al., 2006; Besteiro et al., 2009; Riglar et al., 2011; Srinivasan et al., 2011). Together, these data support a model whereby zoites secrete essential ligands and their corresponding receptors from distinct organelles to assemble their own machinery for host cell entry.

2.2. Recent challenges to the current model of the MJ

Recently, two major studies have emerged that command a reassessment of the notion that AMA1 plays a universal and essential role in the MJ. Giovannini et al. (2011) targeted the gene encoding AMA1 in *T. gondii* and *Plasmodium berghei* via stage-specific deletion of the 3' untranslated region using a recombinase system. This showed that tachyzoites and sporozoites, but not merozoites, were still able to invade host cells and appeared to form normal MJs. Conversely, disruption of RON4 by the same method completely inhibited invasion in all instances. Together this data suggested that AMA1 is not a functional component of the MJ in tachyzoites or sporozoites, whereas RON4 is essential to the MJ. It is important to note, however, that this gene targeting approach is unlikely to ablate expression as the open reading frame remains and is able to utilise downstream transcriptional termination signals, causing a knockdown effect. It is possible that AMA1 is present in vast excess, as is apparent by the minor peripherally located

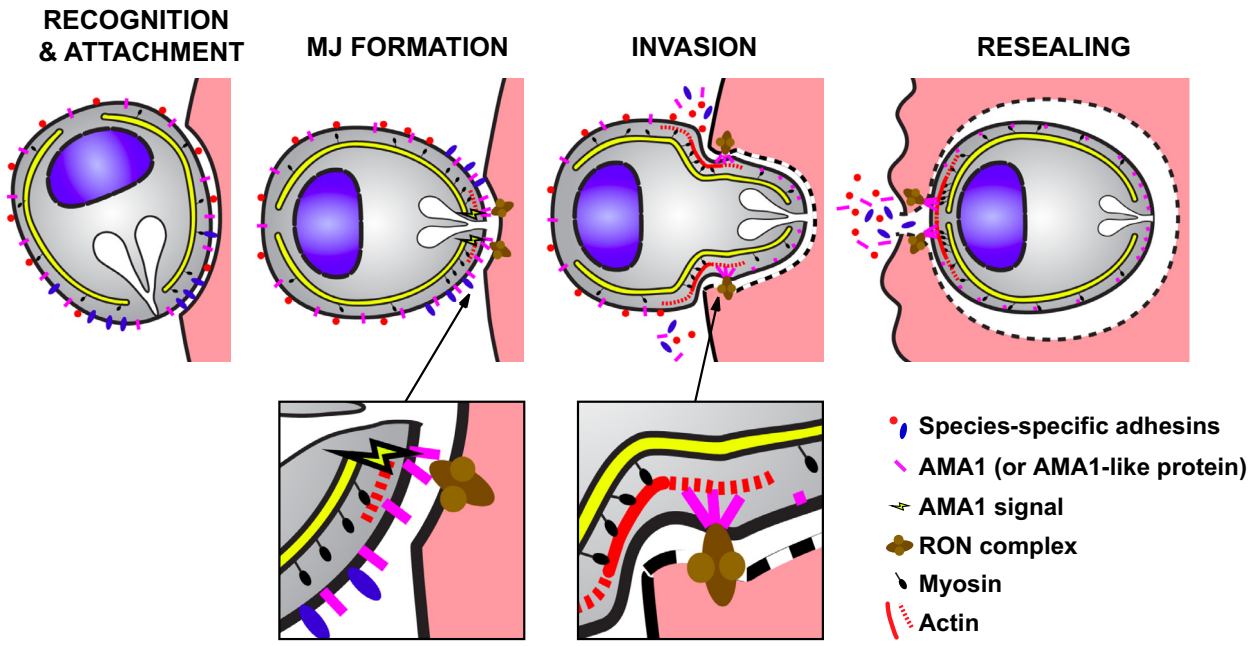

Fig. 1. Putative mechanism of invasion by apicomplexan parasites showing erythrocyte invasion by a merozoite. Host cell recognition, specific attachment and reorientation are likely mediated by several low- and high-affinity binding adhesins that dictate host cell specificity. When apically juxtaposed, the rhoptry neck-derived (RON) protein complex can be translocated into the host cell to act as a receptor for Apical Membrane Antigen 1 (AMA1; or an AMA1-like protein) at the apical tip. This interaction allows intimate contact between the host and parasite (moving junction (MJ) formation), and provides a strong anchor point on the host cell. During invasion, the actomyosin motor utilises the MJ as a traction point to drive penetration. The AMA1 cytoplasmic domain could play a direct role in connecting to the invasion motor or act as a signaling component to coordinate this process. After the zoite has gained entry into the host cell, the MJ may also help to reseal the surrounding host and vacuolar membranes. We speculate that parasites completely lacking AMA1 fail to form a MJ and remain attached to the host cell, but reduced levels of AMA1 allow invasion and instead prevent resealing and subsequent intracellular development.

fraction on zoite surfaces (Thomas et al., 1990; Howell et al., 2003; Riglar et al., 2011). In the case of larger tachyzoites and sporozoites, knockdown could leave a critical but undetectable fraction to maintain functionality, as also deduced by Mital et al. (2005), whereas smaller merozoites may already express a near-critical amount and not tolerate such a knockdown. RON proteins, however, appear to be present in small amounts and may be limiting in all zoites. Thus these observations are likely due to inefficient ablation of AMA1 and consequently do not provide sufficient evidence to reject the current model of AMA1-RON complex binding at the MJ.

Subsequently, Bargieri et al. (2013) attempted to create a complete knockout of AMA1 in *T. gondii* using a conditional DiCre-loxP system and in *P. berghei* by direct homologous replacement. Using a flow cytometry-based method to distinguish extracellular versus intracellular parasites, they concluded that AMA1-null zoites were capable of invading their respective host cell. However, it is possible that the intracellular parasites did not complete invasion to the point of becoming sealed inside the host cell. This is supported by a recent study by Yap et al. (2014) exploring the kinetics of invasion by live cell imaging of *P. falciparum* merozoites using a revised DiCre-loxP technology to knock down AMA1. Interestingly, a subset of AMA1-deficient merozoites penetrated the erythrocyte and either reversed out or remained internal but did not form a ring stage parasite. In many of the abortive invasions, echinocytosis (transient dehydration of the erythrocyte that typically follows invasion (Gilson and Crabb, 2009)) was significantly prolonged, indicating that AMA1 depletion may also lead to a resealing defect. If this were the case for the zoites observed by Bargieri et al. (2013), the parasites would be unable to proliferate, which is consistent with the authors' inability to obtain a stable clonal *P. berghei* AMA1 knockout line.

It is also difficult to argue this point with respect to *T. gondii*, as tachyzoites express AMA1 orthologues/homologues that could compensate for loss of AMA1. Recently, Lamarque et al. (2014) studied a *Tg*AMA1 'knockout' strain that expressed AMA1 at <0.5% of wild-type levels and invaded at 10% of the wild-type rate. These tachyzoites appeared to form intact MJs and binding assays indicated that the RON complex had bound a second AMA1-like protein called AMA2. Upregulation of AMA2 over time allowed these parasites to evolve a compensatory invasion mechanism using the MJ in the absence of AMA1; after 12 months in continuous culture, the invasion rate increased to 20% that of wild-type parasites. Interestingly, disruption of both AMA1 and AMA2 revealed a third AMA1-like protein called AMA4, demonstrating that *T. gondii* possesses multiple MJ complexes to facilitate invasion. While there are no obvious AMA1 homologues expressed in *Plasmodium* spp., it will be interesting to determine whether other proteins, such as AMA1 paralogue MAEBL (merozoite apical erythrocyte-binding ligand) (Kappe et al., 1998; Blair et al., 2002; Ghai et al., 2002), can complement the function of AMA1 in these species. Where 'AMA1-null' zoites form MJs (Bargieri et al., 2013), it would be interesting to isolate and identify the MJ ligand in these cases.

Overall, two possibilities emerge from these studies. Firstly, as argued by Giovannini et al. (2011) and Bargieri et al. (2013), the current model for AMA1-RON complex binding at the MJ may be incorrect. It is possible that AMA1 is a direct host cell adhesin and not involved in MJ formation. If this is the case, there is presumably an as yet unidentified means by which the MJ is formed. Secondly, a more likely explanation is that AMA1 and the RON complex are indeed integral components of the MJ, and AMA1 functional redundancy and/or inefficient knockout genotypes explain the conflicting phenotypes. As such, the evidence presented thus far does not discount the current paradigm, but it is clear that further approaches are needed to clarify the roles of AMA1 and the MJ.

3. The AMA1 cytoplasmic domain

The roles of the cytoplasmic domains of many parasite adhesins have emerged as a focus of study, particularly with regard to AMA1 which, as a potential component of the host anchoring complex, should also have an important role on the cytoplasmic side. The cytoplasmic domain of AMA1 is highly conserved within apicomplexans, indicative of an important function within these parasites. Deletion of the cytoplasmic domain results in functional inactivation of AMA1 and, while intra-genus complementation can restore function, the tails of other invasion ligands are not complementary (Treeck et al., 2009). In these cases, parasites are unable to invade host cells, which points towards distinctive features within the AMA1 tail that are essential for invasion.

3.1. The AMA1 cytoplasmic domain as an invasion motor connection

Active invasion requires a physical connection between the actomyosin motor of the parasite and the cytoplasmic tail of the ligands that are bound to erythrocyte receptors. It was originally hypothesised that aldolase and/or GAPDH provide a bridge between the invasion motor and the cytoplasmic tails of transmembrane adhesins. Both enzymes bind actin with similar affinities and the cytoplasmic tails of many *T. gondii* and *P. falciparum* adhesins bind to aldolase and/or GAPDH in vitro (Buscaglia et al., 2003; Heiss et al., 2008; Jewett and Sibley, 2003). Structural and mutational studies indicate that binding is mediated by a series of aromatic amino acids within the adhesin tails, which exist in AMA1 and are essential for its function in invasion (Buscaglia et al., 2003; Treeck et al., 2009; Srinivasan et al., 2011). Yet no in vivo evidence has been obtained for the interaction between these adhesins and aldolase or GAPDH. Recently, a study by Shen and Sibley (2014) tested this model using *Tg*AMA1. Mutations of the cytoplasmic domain that disrupt aldolase binding in vitro showed no detrimental effect on invasion. Furthermore, aldolase mutants and aldolase-depleted parasites were only impaired when grown in glucose, which led to a toxic accumulation of fructose-1,6-bisphosphate but displayed normal gliding and invasion in glucose-free medium. These parasites were also shown to not compensate for aldolase by binding to GAPDH. This suggests that aldolase is primarily important for energy metabolism and its previously described role is not due to decreased binding to adhesins. This challenges the current model for apicomplexan invasion and this potential role for the AMA1 tail. It is possible that AMA1 associates with another adhesin that links the MJ complex directly or indirectly via another unidentified bridging molecule to a parasite motor component. Concordant with this is an observation by Angrisano et al. (2012) that actin filaments reside behind and not directly in the plane of the MJ, suggesting that the invasion motor and the architecture of the MJ are discrete entities during invasion.

3.2. The AMA1 cytoplasmic domain as a signalling component

Aside from, or in addition to, providing a physical linkage to the invasion motor, the AMA1 cytoplasmic tail could act as a checkpoint to signal engagement with RON2 on the host cell surface and trigger subsequent internalisation. In the presence of AMA1 binding peptides that mimic RON2 and inhibit invasion, *P. falciparum* merozoites attempt to invade, which is illustrated by a sudden pulling motion on the host cell (Treeck et al., 2009; Richard et al., 2010). This indicates that the motor is engaged, possibly due to signalling of the AMA1 tail upon sensing occupation of the AMA1 binding pocket. Mutations of the *Tg*AMA1 tail lead to abortive invasion characterised by an intact MJ but non-penetrative tachyzoite (Sheiner et al., 2010; Lamarque et al., 2014). It is also possible that

the tail, which remains in the invaded zoite after shedding of the ectodomain (Howell et al., 2003, 2005; Buguliskis et al., 2010; Olivieri et al., 2011), plays a role in coordinating the intracellular replication cycle, although this is yet to be resolved (Santos et al., 2011; Parussini et al., 2012). It has already been shown that phospho-signalling on the *P. falciparum* AMA1 tail plays an important role in the invasion process, specifically phosphorylation of Ser610 by protein kinase A (Treeck et al., 2009; Leykauf et al., 2010). A signalling role is also compatible with the potential observation by Giovannini et al. (2011) that minute amounts of AMA1 are capable of facilitating invasion. Such a signalling event would likely be a critical step to maintain coordination in the stepwise invasion process.

4. Concluding remarks

AMA1 has been recognised as essential to invasion for 15 years (Triglia et al., 2000), yet the specific role that this protein plays remains uncertain. The current model posits that AMA1 has a universal and essential role across apicomplexan parasites, facilitating a key binding event that is independent of host-encoded receptors. This provides an advantage where parasites can maintain invasion efficiency irrespective of the diversity of upstream recognition events. The most recent controversies regarding AMA1 function begin to challenge our perception of this mechanism. However, we conclude that this data is not yet sufficiently robust to call for a reconsideration of the role of AMA1. We propose a model whereby AMA1 is typically expressed in excess and the gradual depletion of AMA1 severely affects the structural integrity of the MJ. Below a threshold level, a penetrative junction is formed with inefficient resealing of the erythrocyte. Further reduction prevents formation of any MJ, leading to the inability of the parasite to penetrate its host cell. Fig. 1 illustrates our proposed mechanism for normal invasion, where AMA1 is central to MJ formation. Elucidating the function of the AMA1 cytoplasmic domain will also provide greater insight into the overall function of this ligand. Whether new studies will elicit a major paradigm shift with respect to the function of AMA1 in the MJ remains to be seen. However, it is clear that continued study of the role of AMA1 and that of other potential MJ proteins is needed to provide a sound understanding of the invasion process of apicomplexan parasites.

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